Molecular Assembly of Proteins and Conjugated Polymers: Toward Development of Biosensors

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A molecular assembly in which a conjugated polymer is interfaced with a photodynamic protein is described. The conjugated polymer, functionalized with biotin, is designed such that it can be physisorbed on or chemically grown off a glass surface. The streptavidin-derivatized protein is immobilized on the biotinylated polymer matrix through the strong biotin–streptavidin interactions. The assembly, built on the surface of an optical fiber or on the inside walls of a glass capillary, forms an integral part of a biosensor for the detection of environmental pollutants such as organophosphorus-based insecticides. The protein in the system can be replaced by any biological macromolecule of interest. We study one specific case, the inhibition of the enzyme alkaline phosphatase. The enzyme catalyzes a reaction producing an intermediate compound that chemiluminesces, and the chemiluminescence signal is monitored to detect and quantify insecticides such as paraoxon and methyl parathion. Preliminary results indicate ppb level detection with response time less than 1 minute.

Key words: biosensor • insecticides • phycoerythrin • alkaline phosphatase • immobilization and chemiluminescence

INTRODUCTION

The phenomenal growth in the field of biosensors is in response to the need for rapid, reliable, reproducible, accurate, and sensitive analyses. Biosensors, in which the design of transducing elements is based on electrochemical, optical, thermal or other types of sensing, have been devised to meet this demand. A number of reviews are available covering the design and application of biosensors.[7,15] The design of a biosensor involves combining a biological element that senses the analyte and generates an appropriate signal, and an optical, thermal, electrical, or electronic element for transducing the signal generated. The focus of this article is on the development of a generic immobilization strategy for creating sensing systems that can be adapted for a number of different applications by simple modifications.

Molecular self-assembly is an attractive method to interface various chemical and biological components in thin films. Earlier, we reported on interfacing photodynamic proteins and lipid molecules at the air–water interface of a Langmuir–Blodgett trough.[11] To accomplish this monolayer assembly, a cassette approach was developed in which a biotinylated phycobiliprotein, phycoerythrin, was attached to a monolayer of biotinylated phosphatidyl ethanolamine via the classical biotin–streptavidin interaction. Phycoerythrin is a water-soluble, highly pigmented protein found in the thylakoid membranes of blue-green algae and cyanobacteria. It is involved in funneling sunlight to the photosynthetic reaction center at greater than 90% efficiency and fluoresces 20 times stronger than fluorescene. Phycoerythrin has a high Stoke's shift of 81 nm with 488-nm excitation, and therefore, is a useful candidate in the design and characterization of a light-sensing element in biosensors. Streptavidin is a tetramer protein that has four identical binding sites for biotin. The binding of biotin to streptavidin is almost irreversible with the binding strength comparable with a covalent bond ($K_a = 1 \times 10^{15} M$).[11] In our cassette methodology, streptavidin represents the intermediary component binding to the biotinylated polymer support and provides binding sites to add any biotinylated macromolecules. At present, the conjugated polymer in the molecular assembly is used as a rugged support for the protein binding. However, its conducting properties may also play a role in signal transduction.[10,13] We are currently exploring the possibility of utilizing the photoconductive characteristics of polythiophenes in a biosensor configuration.

Biosensors are needed to detect pesticides that are toxic, in widespread use and persistent in the biosphere. The organophosphorus (OP)-based insecticides are potentially more toxic toward insects than chlorinated hydrocarbons. The relatively higher solubility (versus chlorinated compounds) of the OP-based insecticides in water poses a threat to aquatic life unless they are hydrolyzed (see Table I). It is therefore essential to monitor the levels of these insecticides in industrial waste waters, in agriculture run-off, and in other environments to determine compliance with EPA reg-

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Table I. Solubility in water of some commonly used insecticides.

Insecticide	Solubility in water at 298 K (ppb)
Chlorinated compounds	
DDT	2.00×10^{-1}
Aldrin	2.70×10^{1}
Heptachlor	6.00×10^{1}
Endrin	1.00×10^{2}
Dieldrin	1.86×10^{2}
Organophosphorus compounds	
Paraoxon	2.40×10^{6}
Malathion	1.45×10^{5}
Methyl parathion	6.00×10^{4}
Parathion	2.40×10^{4}
Diazinon	4.00×10^{4}

ulations as well as efficacy of treatments [EP 1.29/2: p. 43, 1980].

The cellular mechanism of insecticidal action of the OP-based compounds involves inhibition of acetylcholine esterase, a key enzyme for nerve function. The resultant accumulation of acetylcholine, responsible for physiological transmission of nerve impulses, causes paralysis at sufficiently high concentrations.[5] There are also other enzymes (acid or alkaline phosphatases, phosphotriesterase, lipases, and chymotrypsin) that are inhibited by the OP-based insecticides, although not to the same extent as acetylcholine esterase. The common feature in the action of all these enzymes is the hydrolysis of phosphate bonds in the insecticides. Because of strong inhibition and wider substrate specificity, acetylcholine esterase and phosphotriesterase are the enzymes of choice in the construction of biosensors for this class of compounds.

In response to the need to detect OP-based insecticides, a variety of sensors have been reported based on chromatographic, biological, or other techniques to separate, identify and/or quantify the insecticides.[2,8,9,12,14] Among these techniques, biological approaches afford rapid, specific, and sensitive detection as well as compatibility with miniaturized and portable devices. We report here the development of a biosensor using our cassette approach for the detection of OP-based insecticides by immobilizing alkaline phosphatase. The advantage of using alkaline phosphatase–catalyzed reactions is in the potential to generate and detect a chemiluminescence signal. Because no external light source is required to generate the signal, the optical instrumentation becomes relatively simple for the experiments described here. With the use of compact detectors such as the charge coupled device (CCD), the biosensor assembly can be miniaturized and made easily portable.

MATERIALS AND METHODS

An aqueous preparation of streptavidin-conjugated alkaline phosphatase (or Str-AP) was supplied as a part of the Southern-Light Chemiluminescent Detection System by Tropix, Inc. (Bedford, MA). Diethylamine (DEA) and chloro 3-(4-methoxy spiro [1,2-dioxetane-3-2′-tricyclo-[3.3.1.1]-decan]-4-yl) phenyl phosphate (CSPD) were also a part of this system. CSPD was available as a 25 m*M* aqueous solution. Sapphire, a luminescence amplifying material (referred to as enhancer), was also supplied by Tropix, Inc. Magnesium chloride was purchased from Fisher Scientific (Fair Lawn, NJ). Paraoxon was supplied by Sigma Chemical Co. (St. Louis, MO) and malathion, methyl parathion, and diazinon were purchased from PolyScience (Niles, IL). Biotin, chlorodimethyloctadecylsilane (CDOS), and all solvents were purchased from Aldrich Chemicals (Milwaukee, WI). Streptavidin-conjugated phycoerythrin (Str-PE) was purchased from Molecular Probes, Inc. (Eugene, OR). Pure silica optical fibers (250/125/100-μm-diameter jacket/cladding/core) were purchased from SpecTran (Sturbridge, MA). Glass capillaries (100 μL, Accupette Pitets) were supplied by Dade Diagnostics, Inc. (Miami, FL). Chem-Solv detergent (Mallinckrodt Specialty Chemical Co., Paris, KY) was used to clean the glass substrates prior to molecular assembly. Deionized and distilled water was used in all preparations.

Molecular assembly on a glass surface was carried out by first silanizing the glass to generate a hydrophobic surface. Either a glass slide, an optical fiber, or a glass capillary was cleaned using Chem-Solv in an ultrasonic bath. After drying, the glass surface was immersed for 30 min in a 2% solution of CDOS in dry petroleum ether. The surface was then thoroughly rinsed with ether, dried, and stored in a dust-free environment.

The synthesis of biotinylated poly(3-undecyl-co-3-methanol thiophene) (or B-PUMT) has been reported.[6] The polymer was sparingly soluble in chloroform. Molecular assembly on a glass surface involved silanizing the glass surface and immersing in a solution of B-PUMT/chloroform at a concentration of 10 μg/mL. A 24-h incubation was found to be optimal for polymer adsorption. The glass surface was then immersed in an aqueous solution of Str-PE for about 8 h. Finally, the glass surface was rinsed thoroughly in water to remove any nonspecifically bound polymer or protein. The entire process was conducted at ambient conditions. Idealized schematics of the assembly process on an optical fiber and various interactions between a glass surface, polymer, and protein are illustrated in Figure 1a and b.

The molecular assembly of the polymer and the protein on an optical fiber was characterized by recording fluorescence spectra of phycoerythrin. A light source, a photomultiplier tube (PMT) coupled to a monochromator, a photon counter, and a personal computer were used to collect and process the data (Fig. 2). The protein was excited with an Ar^{+} laser (10 mW, 488 nm), and the spectra were recorded between 500 and 667 nm. To detect and measure the chemiluminescence signal generated by the enzyme alkaline phosphatase either immobilized on a glass surface or dissolved in bulk aqueous solution, a simplified version of the optical set-up in Figure 2 was used by eliminating the light source and the monochromator.

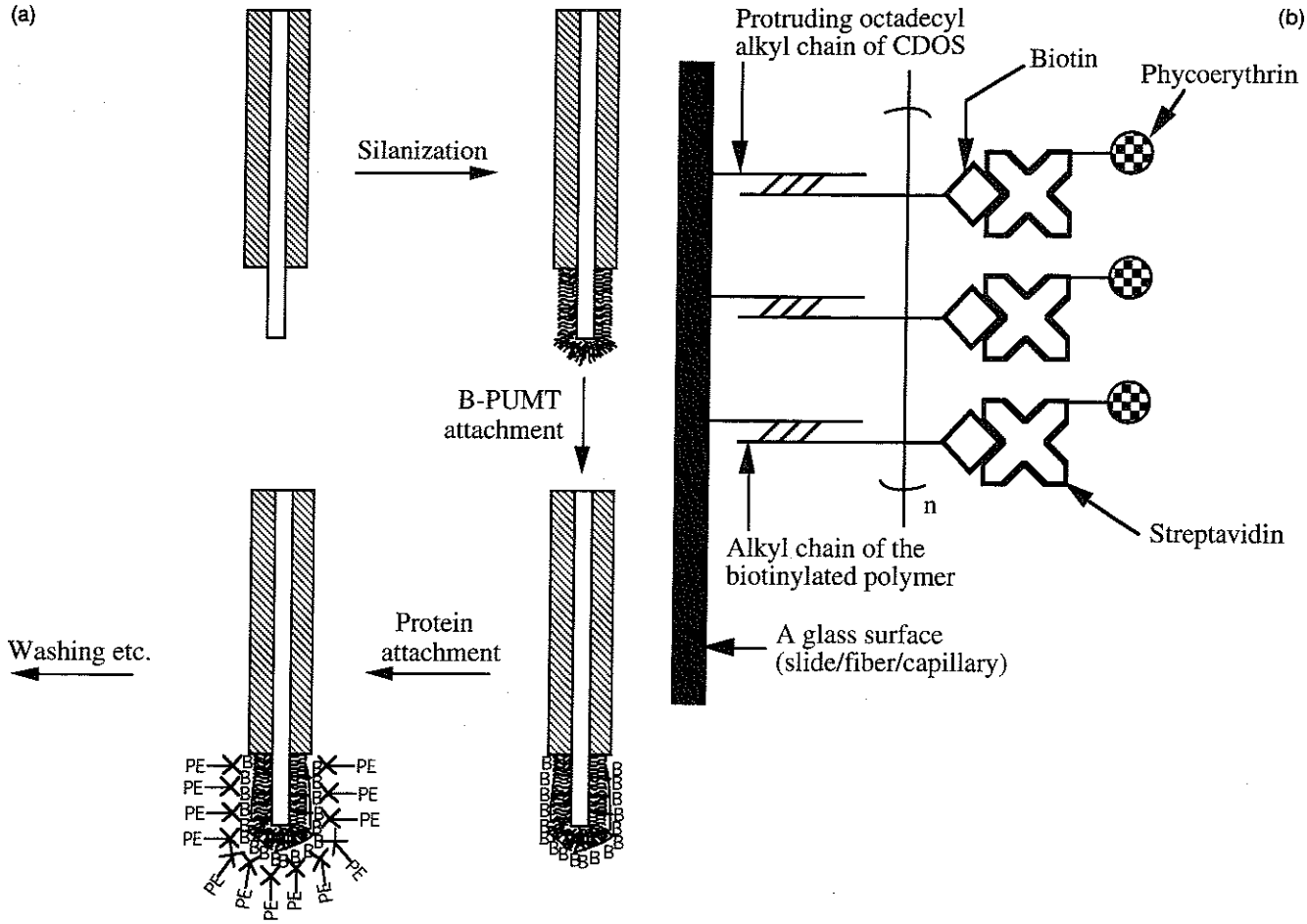

Figure 1. (a) Idealized schematic of polymer/protein assembly on an optical fiber. X–PE = streptavidin-conjugated phycoerythrin, and B = biotin. (b) Idealized schematic of interactions between a glass surface, polymer, and protein.

The reactions with alkaline phosphatase in bulk aqueous solution were carried out in a glass test tube. The test tube was fixed to a stand in front of the PMT, and was held in a given position with the help of markers. The assembly was placed in a dark room, and the PMT was connected to a photon counter via an amplifier located in an ante-room. A solitary test tube was used in a given set of reactions with a given insecticide to eliminate the effect of tube geometry on signal intensity. Less than 10 s elapsed between the initiation of the reaction and the start of data acquisition. Initial slopes were calculated based on the first 20 data points collected in as many seconds.

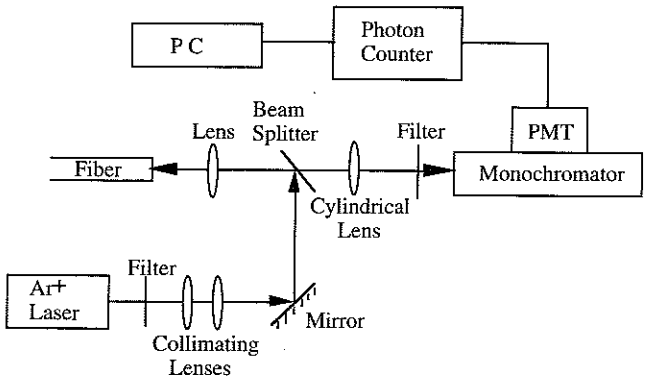

Figure 2. Schematic of experimental set-up for fluorescence measurement from fiber surface.

RESULTS AND DISCUSSION

The molecular assembly and the enzymatic reactions were carried out at room temperature. Phycoerythrin and the polymer were first assembled on a glass slide to establish and optimize the technique for subsequent immobilization on an optical fiber surface as well as to measure film thicknesses using ellipsometry.[1,11] Figure 3 shows the fluorescence signal from phycoerythrin immobilized on an optical fiber surface. The control with unsilanized fiber showed near-background signal intensity implying that the binding of phycoerythrin via hydrophobic interactions between the polymer backbone and the silanized fiber surface was both specific and strong enough to withstand mild washing required to remove nonspecific protein binding.

To develop a biosensor for the detection of organophosphorus insecticides, the model protein, phycoerythrin, in

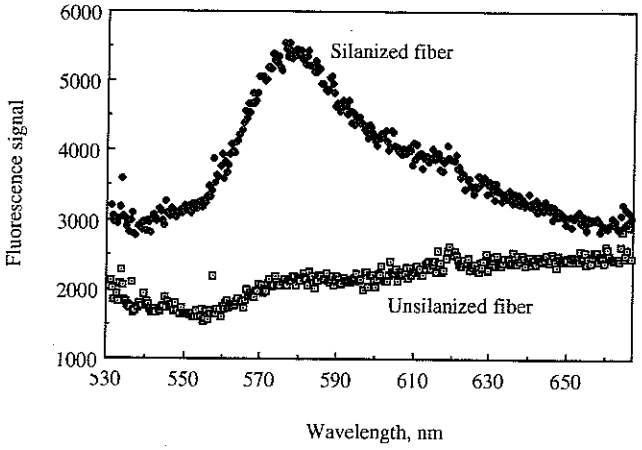

Figure 3. Fluorescence spectra of phycoerythrin immobilized on fiber surface.

the sensing element was replaced with alkaline phosphatase. First, the enzyme kinetics were studied in bulk aqueous solutions in the presence and absence of the insecticides. These kinetic data will be discussed in detail elsewhere (manuscript in preparation). Table II presents some data on the effect of the substrate and inhibitor concentrations on the insecticide detection limit for alkaline phosphatase in solution. Although the phenomenon indicates that the inhibitor competitively binds to the enzyme, this effect could be used in an advantageous manner in the sensor design. Clearly, the lower the CSPD concentration, the better the detection limit. Therefore, the detection limit can be controlled by controlling the CSPD concentration up to a point where the molar ratio of CSPD to inhibitor falls in the range of 1.3 to 2.2 (see Table II), or where the signal to noise ratio becomes intolerable, whichever occurs first. Figure 4 shows a linear decrease of initial reaction rates as a function of inhibitor concentration for one of the most widely used OP-based insecticides, paraoxon. Although three different insecticides were analyzed, data is presented in this study for only paraoxon as a model compound. Detection levels of 50 and 75 ppb were achieved for paraoxon and another commonly used insecticide, methyl parathion, respectively, with alkaline phosphatase in bulk aqueous phase. More importantly, this method allows very rapid detection of insecticides, less than 30 s. Table III gives a comparison of the detection limits and speeds for different methods of paraoxon quantification reported in the literature as well as the method reported here.

Table II. Paraoxon detection limit as a function of CSPD concentration.

[CSPD] (μM)	Paraoxon detection limit (ppm)	[CSPD]/[paraoxon] in reaction mixture
290	50	1.6
1.82	0.4	1.3
0.874	0.11	2.2
0.290	0.05	1.6

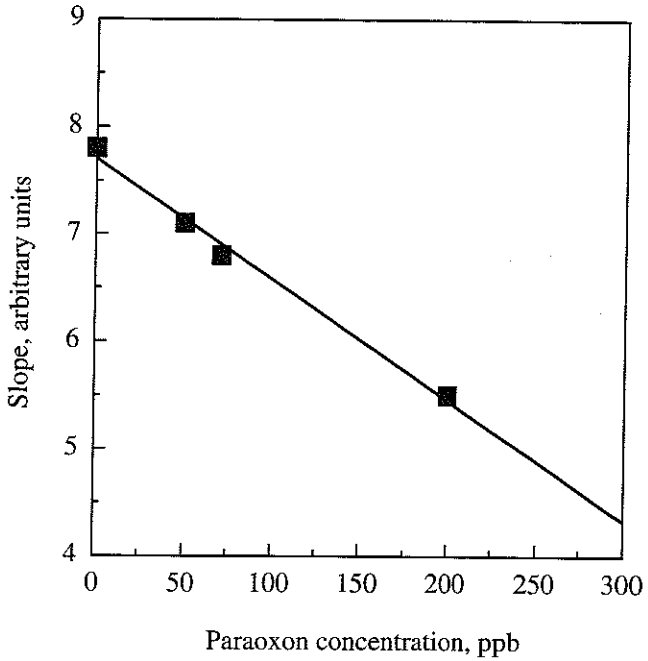

Figure 4. Calibration curve for paraoxon with alkaline phosphatase in bulk aqueous phase: 0.1 *M* DEA; 1 m*M* $MgCl_2$, 10% enhancer; 0.29 μM CSPD; and 0.14 n*M* enzyme.

Alkaline phosphatase was next immobilized on the inside walls of a glass capillary. The rationale for using a glass capillary was the availability of large surface area, its ease of handling, and its capability to hold a finite microvolume of the reaction mixture. The enzyme and the polymer were assembled on the inside walls of capillary as described in Materials and Methods. Controls showed no chemiluminescence signal and therefore no adherence of the polymer and protein to the unsilanized glass surface was presumed. The stability of alkaline phosphatase was ascertained by measuring the activity of the enzyme, immobilized on a single glass capillary, a number of times over a period of 1 month. About 80% of the enzyme activity was retained during this period, and the result was reproducible (Fig. 5). The sensing element, therefore, can be reused many times, an important characteristic of a biosensor. The capillary assembly was used to generate calibration curves for the insecticides. Figure 6 illustrates a calibration curve for paraoxon. About 1 ppm of paraoxon was detected with this system, and current work is focused on lowering the detection limit to ppb levels by enhancing the signal collection efficiency.

The methodology for protein immobilization and the technique for insecticide detection hold promise in the development of a sensitive, rapid, compact, portable, and relatively inexpensive biosensor device for OP-based insecticide detection. In the present scheme, a target biomolecule can be potentially incorporated into the polymer matrix by replacing phycoerythrin in Figure 1. In addition, other enzyme systems such as phosphotriesterases that have a broader substrate specificity[3,4] will be used for chemiluminescence detection. A broader pH range could be covered

Table III. Features of different methodologies for paraoxon detection.

Detection mode	Paraoxon detection limit (ppb)	Response time (min)	Reference
Amperometric	1–10	30–120	Palleschi et al. (1992)[9]
Absorption	55	21	Trettnak et al. (1993)[14]
Chromatographic	<1	10–15	Martinez et al. (1992)[8]
Chemiluminescence	50	<0.5	Current study

between acid and alkaline phosphatases to detect a variety of insecticides.

In another approach, poly(3-ethanolthiophene) was grown off the fiber surface using a bifunctional silane compound. After silanizing the glass surface, a thiophene monomer was attached to the free end of the silane compound through an amide linkage. Polymerization was subsequently carried out in the presence of 3-ethanolthiophene at a number of such reactive centers over the fiber surface, and the result was a brushlike polymer on the fiber. The polymer was subsequently biotinylated at the ethanol moieties. The advantage of this methodology over the hydrophobic adsorption of prepolymerized B-PUMT is the ability of the covalent attachment strategy to control the extent of polymerization and/or the extent of biotinylation, which in turn controls the extent of protein binding. Again, the efficacy of this attachment methodology was demonstrated with the model system of streptavidin-conjugated phycoerythrin.[1]

CONCLUSIONS

Methodologies to interface conjugated polymers and biological macromolecules are discussed. The methodologies were characterized using a model photodynamic protein, phycoerythrin. This generic approach was used to create molecular assemblies for biosensors for the detection of organophosphorus-based insecticides. Alkaline phosphatase–catalyzed chemiluminescence signal generation was used to establish detection limits and calibration curves for two insecticides. Rapid and sensitive detection of 50-ppb paraoxon and 75-ppb methyl parathion was achieved in bulk aqueous solutions. This chemiluminescence-based technique allowed rapid determination of pesticide concentration in less than 30 s, at least 20 times faster than values reported in the literature. The detection levels could be further lowered by reducing the CSPD concentration. Experiments with other OP-based insecticides show that the technique is general to all of OP-based insecticides tested to date.

The system with immobilized enzyme was shown to be reusable a number of times without significant loss of enzyme activity for insecticide detection. Future studies are focused on lowering the detection limits, optimizing the immobilization strategy, expanding the utility of the device to cover a variety of pesticides, and assessing potential interferences in environmental samples. A flow injection sys-

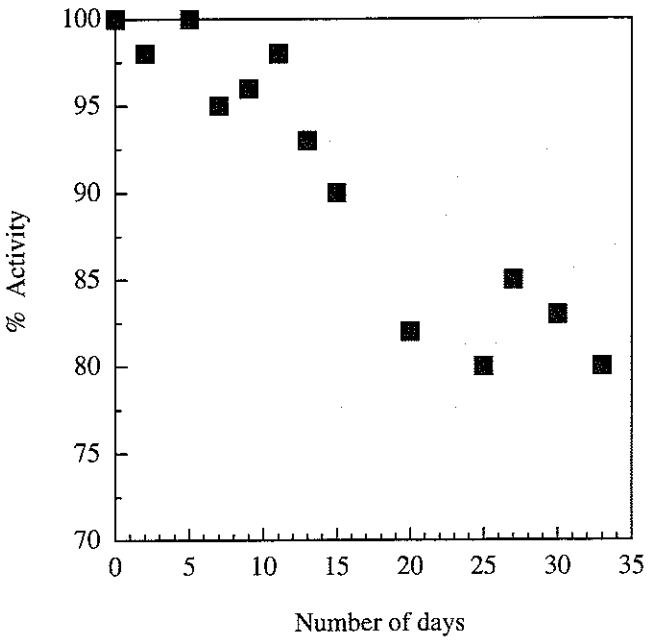

Figure 5. Stability of alkaline phosphatase immobilized on the inside walls of a glass capillary: 0.1 *M* DEA; 1 m*M* $MgCl_2$; 10% enhancer; 0.4 m*M* CSPD.

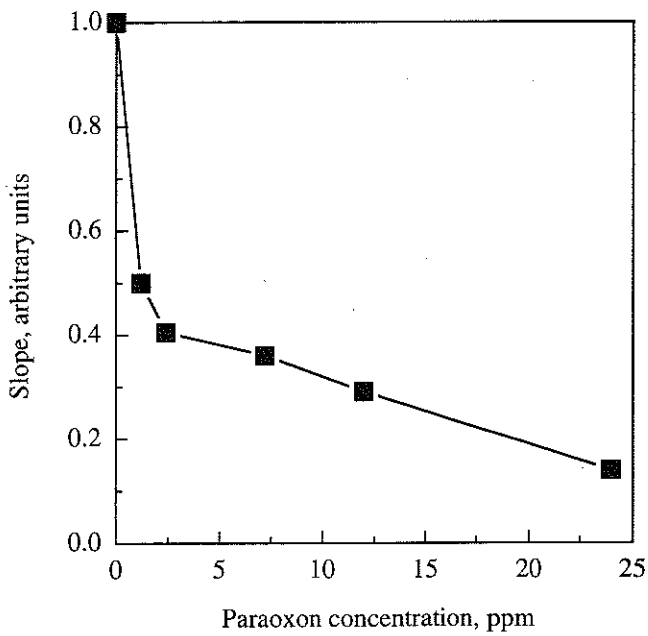

Figure 6. Calibration curve for paraoxon with the enzyme immobilized on the inside walls of a glass capillary: 0.1 *M* DEA; 1 m*M* $MgCl_2$; 10% enhancer; and 0.2 m*M* CSPD.

tem is being developed using glass capillaries or optical fibers for continuous operation.

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